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GRANT NUMBER DAMD17-97-1-7109

TITLE: A Modulator of FGF's in Breast Cancer

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CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20007

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE

A Modulator of FGF's in Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7109

6. AUTHOR(S)

Harris, Violaine K.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgetown University Medical Center
Washington, DC 20007

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

19990301008

13. ABSTRACT *(Maximum 200 words)*

The growth of new blood vessels, or angiogenesis, is an important part of breast cancer biology. We have found that a secreted binding protein for fibroblast growth factors (FGF-BP) is expressed in many breast cancer cell lines and primary breast tumor samples. In addition, FGF-BP is expressed in squamous cell carcinoma and colon cancer and modulation of FGF-BP expression in these tumor types has a significant effect on tumor growth and angiogenesis. In order to better understand the regulation of FGF-BP and how its aberrant expression might lead to activation of angiogenic pathways, I isolated the human FGF-BP promoter and determined which functional promoter elements were necessary for its expression. In particular, I found that the FGF-BP gene is transcriptionally up-regulated by the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) which activates the protein kinase C (PKC) pathway. The positive regulatory elements which mediate FGF-BP induction by TPA include a juxtaposed Ets/AP-1 site as well as a C/EBP site. Furthermore, the presence of a distinct repressor element was detected which normally limits the response of the promoter to TPA. Additionally, I found that the FGF-BP is induced in the presence of EGF (epidermal growth factor), which is known to play an important role in the pathogenesis of breast cancer.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Annual report for Grant Number DAMD17-97-1-7109

P.I. Violaine K. Harris

Title: A Modulator of FGF's in Breast Cancer

I. Introduction

Growth factors, tumor angiogenesis and metastasis

One of the pivotal roles of locally-acting polypeptide growth factors is the induction of new blood vessels in a healing wound as well as in growing tumors. It has been shown in numerous studies with different approaches that a solid tumor cannot grow beyond a few millimeters in size without sufficient blood supply. In addition to the nourishing function of tumor blood vessels, they provide a pathway for the tumor cells to metastasize to distant organs (1-5). A direct correlation between blood vessel density in primary tumors and their metastasis has been reported for breast cancer (6-10). Most interestingly tumor angiogenesis as reflected in microvessel density is an independent prognostic indicator in breast cancer patients when tested against known parameters (e.g. tumor size, estrogen receptor, lymph node status, c-erbB-2 expression). Since angiogenesis is such an important feature of *in vivo* tumor biology, the driving forces behind this process need to be understood.

The role of fibroblast growth factors (FGFs) and a potential function for a novel binding protein (BP) for FGF

The most prominent and best studied angiogenesis factors are members of the fibroblast growth factor (FGF) family of polypeptides (11,12). Several members of the FGF family, mainly bFGF and aFGF are very effective angiogenesis factors and have thus been a focus of research in tumor vascularization in the past decade. The biological activities of both FGFs can be quenched by tight binding to heparansulfate proteoglycans present in the extracellular matrix (13-16). It is only partly understood how these FGFs become solubilized and thus activated in embryonic or in tumor tissues that require angiogenesis for their growth. One established mechanism that can solubilize bFGF from this storage site is the digestion of the glycosaminoglycan portion of the cell attachment molecule by heparanases (17-20).

An alternate mode of delivering active FGF from the storage site to its receptor could be binding to a secreted carrier protein. Recently, a secreted protein has been described binds to aFGF and bFGF in a non-covalent, reversible manner (21). Furthermore, bFGF bound to this protein was prevented from degradation and retained its mitogenic activity (21). These characteristics make this **FGF-binding protein (BP)** an excellent candidate carrier molecule for FGFs. We hypothesized that this BP could be an important regulator that releases immobilized FGFs from their matrix storage site and thus activates them *in vivo* (22,23).

The secreted binding protein for FGF (BP) as a carrier for immobilized FGFs

Recently, it was demonstrated by our laboratory that expression of this binding protein in cell lines which express bFGF leads to a tumorigenic and angiogenic phenotype of these cells (22). We also showed that BP-transfected cells release the protein into their media together with bFGF in a non-covalently bound form. This released bFGF becomes activated biologically. In addition, *in vivo* tumor growth of a BP-positive squamous cell carcinoma (SCC) cell line and a colon cancer cell line was inhibited by a reduction of endogenous BP using BP-targeted ribozymes (24). This supports the notion of an activating step for the locally stored bFGF due to expression and secretion of BP.

BP mRNA is expressed in tumor cell lines and primary tumor tissue of squamous cell carcinomas, colon cancers, and breast cancers (22). Using skin carcinogenesis as a model for epithelial cancers, we studied the role of BP expression during tumor progression. We found that BP mRNA is upregulated in the skin during development but drops to low levels in the adult mouse skin. Interestingly, in both mouse and human skin, BP mRNA and protein levels increase more than 3-fold upon treatment with the PKC-activating agent TPA (12-O-tetradecanoylphorbol-13-acetate), and is further upregulated (4-7 fold) in DMBA/TPA induced papillomas and carcinomas (25,26). The correlation between BP expression and tumor promotion by TPA strongly suggests a role for BP in tumorigenesis. The study of BP regulation in the mouse skin, which I co-authored, was published last year (25).

Using RT-PCR to screen for FGF-BP expression, we found that FGF-BP is expressed in a 4 out of 6 clinical samples of primary human breast cancers, and in 9 out of 14 breast cancer cell lines. We also detected BP

mRNA expression in the mammary gland of the the human and the mouse. This pattern of expression suggests that BP may be involved in breast cancer progression, where its deregulated expression could potentially contribute to tumor growth and angiogenesis. During my thesis research I plan experiments that will test the role of BP in human breast cancer cell progression, and its mechanism of regulation by the tumor promoter TPA.

The aims of my grant application were the following:
Aim 1: To study the tumor growth effects of BP expression in breast cancer cells. and **Aim 2:** To study the mechanisms of regulation of BP by Protein Kinase C (PKC).

II. Summary of Results

The results regarding Aim 2 will be addressed in this report. This work, for which I was first author, has previously been published in the **Journal of Biological Chemistry in July 1998** (27). In order to investigate the mechanism of TPA induction of FGF-BP gene expression, we examined the level of FGF-BP mRNA after treatment in the ME-180 cell line, which expresses high levels of endogenous FGF-BP. We found that TPA increased FGF-BP mRNA levels in a time- and dose-dependent manner. Furthermore, induction was mediated *via* the PKC signal transduction pathway, since treating with a specific PKC inhibitor, Calphostin C, blocked the effect of TPA. Results from actinomycin D and cycloheximide experiments as well as nuclear transcription assays revealed that TPA upregulated the steady-state levels of FGF-BP mRNA by increasing its rate of gene transcription independently of *de novo* protein synthesis.

Isolation and Characterization of the Human FGF-BP Promoter. In order to better understand the transcriptional regulation of the human FGF-BP gene, 1.8kb of genomic sequence upstream to the known 5'UTR sequence of human FGF-BP cDNA was isolated from a human genomic library and sequenced and the transcription start site of the human gene was determined using primer extension analysis. In addition, alignment between the human and mouse FGF-BP promoter which we cloned previously (25) revealed a region of high homology with 70% nucleotide identity within the first 200 nucleotides upstream from the transcription start. Nucleotide homology dropped significantly in more upstream sequences, suggesting that the proximal conserved 200 nucleotides of the promoter could be important for transcriptional regulation of FGF-BP in both species.

Sequence analysis of the promoter demonstrated the presence of numerous consensus transcription factor binding sites which were conserved between mouse and human FGF-BP promoters and which may have functional importance in FGF-BP regulation. Consensus binding sites included TATA box is located at about -25 upstream from the transcription start for both promoters. Also, a highly conserved consensus binding site for C/EBPß, a member of the CCAAT/enhancer binding protein (C/EBP) family of leucine zipper transcription factors which plays a central role in the acute-phase response and in a number of cell differentiation pathways (28-30). An AP-1 consensus binding site lies juxtaposed to a sequence with homology to an Ets factor binding motif, suggesting potential functional similarity to the juxtaposed Ets/AP-1 site found genes involved in invasion and metastasis such as collagenase and urokinase plasminogen activator (uPA) (31,32). In addition, a consensus Sp1 factor binding site, an additional Ets factor binding motif, and a potential NF-κB binding site are located in the conserved region of the promoter and may play a role in transcriptional regulation of FGF-BP as well.

Functional analysis of the human FGF-BP promoter. To identify the functional promoter elements involved in FGF-BP gene regulation by TPA, progressive 5' deletion mutants as well as internal deletion mutants were constructed based on the location of consensus factor binding sites on the promoter. Deletion constructs were transiently transfected into ME-180 cells and their relative luciferase activity was assayed in the absence or presence of TPA. Basal activity of the FGF-BP promoter was mediated by a combination of the Sp1 site, as well as by the juxtaposed Ets/AP-1 site.

The regulatory elements mediating TPA induction of the promoter were all contained in the first 118 base pairs upstream of the tranascription start site. Further mutational analysis revealed that full TPA induction required an interplay between several regulatory elements, including the Ets/AP-1 site and the C/EBP site.

TPA regulation of the FGF-BP promoter involves a repressor element juxtaposed to the AP-1 site. Between the AP-1 site and the C/EBPß site lies a region of low homology between the human and mouse BP promoter sequences. Because this region was not suspected to have any effect on TPA induction, an internal deletion removing this region (-57 to -47) was tested as a control. Surprisingly, in the Δ57/47 construct. TPA induction of the FGF-BP promoter increased from approximately 5- fold to 11-fold, suggesting the presence of a possible repressor which may interact with this site. The lack of sequence conservation between the human

and mouse in this region may reflect a difference in the regulation of FGF-BP between the two species. The -57 to -47 deletion disrupts an AACGTG (-60 to -55) which is juxtaposed to the 3' end of the AP-1 site and which shows some similarity to the CACGTG E-box element recognized by a number of helix-loop-helix factors (33). To test this imperfect E-box for repressor activity, a C to T point mutation at position -58 was introduced into the -118/+62 BP promoter **construct. The m-58 construct showed a dramatic increase in TPA induction up to 16-fold above background. This data shows that the point mutation at position -58, as well as the internal deletion from -57 to -47, disrupts repression of the FGF-BP promoter which normally limits the response to TPA.**

Transcription factor binding to FGF-BP promoter elements. In order to ascertain that TPA-induction of FGF-BP was due to direct activation by transcription factors, we performed gel retardation analysis to show transcription factor binding to FGF-BP promoter elements. This analysis showed specific and TPA-inducible protein binding to the Ets, AP-1, and C/EBP sites. Furthermore, distinct, specific and TPA-inducible binding to the imperfect E box repressor element was also apparent. **Overall, this data indicated that TPA effects on FGF-BP gene transcription are tightly controlled by a complex interplay of positive elements and a novel negative regulatory element (27).**

EGF induction of the FGF-BP gene. During the course of these experiments, we found that both fetal bovine serum (FBS) and the epidermal growth factor (EGF) can regulate FGF-BP expression. Using Northern blot analysis to detect endogenous levels of FGF-BP mRNA, we found that EGF and FBS treatment causes a rapid induction of FGF-BP gene expression (unpublished results). EGF induction of FGF-BP was not dependent on new protein synthesis, suggesting that this is a direct effect on the FGF-BP gene. Furthermore, EGF and FBS induction were dependent on the PKC pathway, since treatment with a PKC inhibitor blocked their effects. Preliminary analysis of the FGF-BP promoter has shown that some of the similar transcriptional elements (i.e. Ets/AP-1 and C/EBP) are required for full induction of the promoter by EGF. Regulation of FGF-BP by EGF is highly significant to breast cancer. The EGF family of growth factors plays an important role in the development of the mammary gland and in the pathogenesis of breast cancer.

III. Publications and Abstracts

Harris, V.K., Liaudet-Coopman, E.D.E., Boyle, B.J., Wellstein, A., Riegel, A.T. Phorbol ester-induced transcription of a FGF-binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements. *J Biol Chem* 273: 19130-19139. 1998 July.

Harris, V.K., Liaudet-Coopman, E., Wellstein, A., Riegel, A.T. A fibroblast growth factor binding protein (FGF-BP) is transcriptionally regulated by phorbol esters and retinoic acid. *Proceedings of the American Association for Cancer Research*, 89th Annual Meeting, New Orleans, LA, March 28-April 1, 1998

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